

General Thoracic Surgery

Replacement of a tracheal defect with a tissue-engineered prosthesis: Early results from animal experiments

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Objectives: The major problems in the development of tracheal prosthesis are anastomotic dehiscence and stenosis, caused by poor epithelialization of the prosthetic graft. We developed a novel tracheal prosthesis with viable mucosa transplanted from the oral cavity and reported excellent long-term results after thoracic tracheal replacements in dogs. In the current study, we used tissue-engineering techniques to construct a mucosal prosthetic lining from skin cells and evaluated its usefulness in tracheal replacement.

Methods: Abdominal skin patches (5 × 10 cm) were harvested from 10 adult mongrel dogs. The epithelial cells were separated, cultured in vitro for 4 weeks, and then seeded onto a porous polylactic glycolic acid scaffold (6 × 8 cm) to construct a lining mucosa. This was then mounted onto the prosthesis framework, made with polypropylene mesh reinforced with polypropylene rings. The mucosa-lined prosthesis was wrapped with the greater omentum of the same dog and placed in the peritoneal cavity for 1 week. Complete surgical resection and replacement of a thoracic tracheal segment (5 cm in length, just above the carina) was then performed using the prosthesis.

Results: The animals regained full activity and survived with normal activity. Bronchoscopy at 1 week and at 1 and 2 months revealed no stenosis in the anastomosis.

Conclusions: This highly biocompatible tracheal prosthesis could prove useful for the reconstruction of large, circumferential tracheal defects.

The reconstruction of large, circumferential tracheal defects remains a challenging problem in tracheal surgery. Many types of prosthetic grafts and tissue grafts have been used in attempts to repair such defects but with limited success because of graft ischemia and immune rejection leading to anastomotic dehiscence and stenosis. The prosthetic trachea is poorly epithelialized and subject to chronic infections, which stimulate granular tissue formation, anastomotic disruption, and erosion of the major blood vessels.¹⁻⁷

We developed a novel tracheal prosthesis with viable mucosa and reported excellent long-term results after using it for thoracic tracheal replacements in

dogs.^{8,9} The prosthesis has a basic framework made with high-porous synthetic material to promote ingrowths of new vessel. The luminal surface is lined with autogenous mucosa from the oral cavity to promote epithelialization of the graft, and biodegradable glues such as gelatin are used to enhance an airtight attachment between tissue and the prosthesis. We initially transplanted the graft into the greater omentum to promote vascularization of the graft and to maintain the viability of the implanted mucosa. We found that after 6 months the prostheses were completely incorporated by the host trachea in all dogs and confluent epithelialization was confirmed histologically from the upper to the lower anastomotic site of the prosthesis. Furthermore, the transplanted mucosal cell types had changed to ciliated columnar epithelium.

In this study, aimed at constructing a readily usable artificial trachea, we used tissue-engineering techniques to make a prosthetic lining mucosa from skin epithelium and evaluated its usefulness for tracheal replacement.

Materials and Methods

Animals

The animals used for this study were 10 adult mongrel dogs, weighing 15 to 20 kg. The dogs were acclimated for 7 days before experiments. They were individually housed and maintained at an environmental temperature of 22° ± 2°C and on a 12/12-hour light/dark cycle. They were fed a canine diet (Purina Korea Co, Seoul, Korea) with water ad libitum. All animals received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health and The Animal Experiment Guidelines of Samsung Biomedical Research Institute. The experiment is summarized in Table 1.

Preparation of the Prosthesis

The construction of the prosthesis has been described previously.^{8,9} Ten polypropylene rings (diameter 30 mm; thickness 1 mm; width 1 mm) were attached at 5-mm intervals to the external surface of an open-ended cylinder made of polypropylene mesh (Ethicon, Somerville, NJ), using thermal melt bonding. Type B bovine gelatin (mean molecular weight 23,000; Sigma, St Louis, Mo), which does not block the diffusion of oxygen and nutrients from omental blood vessels, was used to make an adhesive surface for the oral mucosa and omentum. The prosthesis was air-dried and then sterilized using ethylene oxide gas (Figure 1).

Preparation of Mucosa from Skin Epithelium by Tissue Engineering

Porous polymer scaffolds were made from an 85:15 copolymer of poly (D,L-lactide-co-glycolide) (PLGA; Polysorb; Alkermes, Wilmington, Ohio), a white to nearly white powder with an average molecular weight of 110,000 Da. Scaffolds were prepared using a particulate-leaching technique. The polymer was dissolved in chloroform (Sigma) to yield a 10% solution, and sieved gelatin particles (Sigma) were added. The vortexed dispersion was cast in a Teflon container (5 × 5 cm) (Daelim, Seoul, Korea), followed by

TABLE 1. Schematic presentation of animal experiment for the development of tissue-engineered tracheal prosthesis

Animal no.	Cx peritoneum*	Cx trachea†	Bronchoscopic evaluation		
			1 week	1 month	2 month
Dog 1	None	None	Edema	Good	Good
Dog 2	None	Pneumothorax	Edema	Hypertrophy	Good
Dog 3	None	None	Edema	Good	Good
Dog 4	None	None	Edema	Good	Good
Dog 5	Evisceration‡	Not done	Not done	Not done	Not done
Dog 6	None	Pneumothorax	Good	Good	Good
Dog 7	None	None	Edema	Hypertrophy	Good
Dog 8	None	None	Edema	Hypertrophy	Good
Dog 9	None	None	Good	Good	Good
Dog 10	None	None	Edema	Good	Good

The animals were numbered in order of experiment. Every animal with intrathoracic tracheal replacement survived and will be killed for the systematic gross and microscopic examination after 6 months of observation. Bronchoscopic evaluations were performed after intrathoracic tracheal replacement. Hypertrophy of the anastomosis, especially at the proximal anastomosis, was noticed in some animals.

*Complication after transplantation of the prosthesis into the peritoneal cavity for in vivo culture.

†Complication after intrathoracic tracheal replacement.

‡At 5 days after the intraperitoneal placement of the prosthesis, bowel protrusion through the abdominal wound was noticed. Intrathoracic tracheal replacement was not performed in this animal because of the risk of graft infection.

vacuum drying for 48 hours. Scaffolds were annealed at a temperature of 120°C in a drying oven (Chang Shin Scientific Co, Seoul, Korea). The scaffold was then immersed in deionized water for 48 hours to leach out any particles and freeze-dried under a vacuum for 24 hours. All scaffolds were stored under vacuum until used.

Cell Preparations

For cell recovery and culture, adult mongrel dogs weighing 15 to 20 kg were anesthetized with intravenous thiopental sodium (10-15 mg/kg) and immobilized in a supine position. After injection of vecuronium bromide (0.1 mg/kg), the dogs were intubated orally using a cuffed endotracheal tube (inner diameter 6.5 mm) connected to a respirator (Narkomed model 2B; Dräger North America, Minster, Ohio). Anesthesia was maintained by inhalation of a gas mix (O₂ 1 L/min; N₂O 1 L/min; halothane 1%-1.5%). The operative area was shaved and disinfected. An abdominal skin patch (5 × 10 cm) was harvested, cut into small pieces after removal of the subcutaneous fatty tissues, and washed twice in phosphate-buffered saline solution (PBS) containing antibiotics. Tissues were immersed in 0.02% dispase (Gibco-BRL, Life Technologies, Gaithersburg, Md) solution for 16 hours at 4°C. Dispase was prepared under sterile conditions 1 day before surgery by diluting it with sterile PBS to 50 μg/0.05 mL (pH = 7.4). They were then treated with 0.1% trypsin solution for 10 minutes at 37°C to separate the cells. Enzyme activity was stopped by adding Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, and the suspension was filtered to remove undigested tissue. The suspension was centrifuged for 5 minutes at

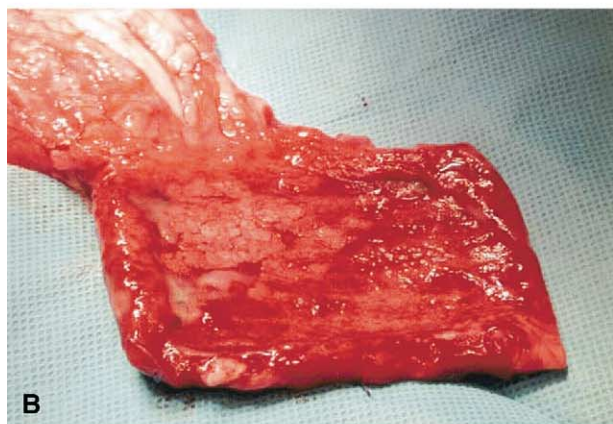
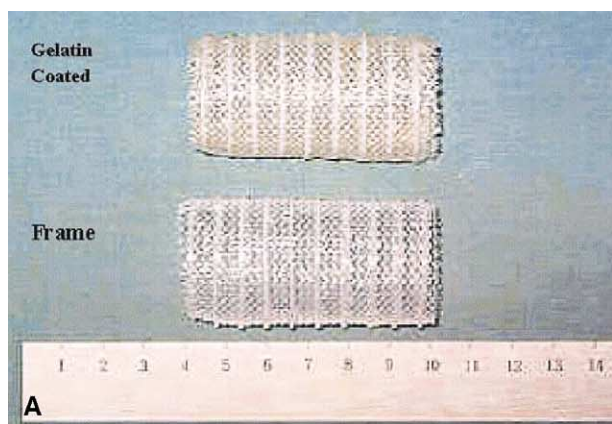


Figure 1. A, Tracheal prosthesis made from polypropylene mesh and polypropylene rings, before (*Frame*) and after gelatin coating (*Gelatin Coated*). B, Tissue-engineered mucosa from skin epithelium on the greater omentum. C, Tracheal prosthesis after 1 week of implantation. Tissue-engineered mucosa, which lined the inner surface of the prosthesis, tightly connected to the omental wrapping.

1500 rpm, and the cell pellet was resuspended in full keratinocyte culture medium (Gibco-BRL).

To plate the scaffolds, cell numbers and viability were quantified by trypan blue vital dye staining and counted using a hemo-

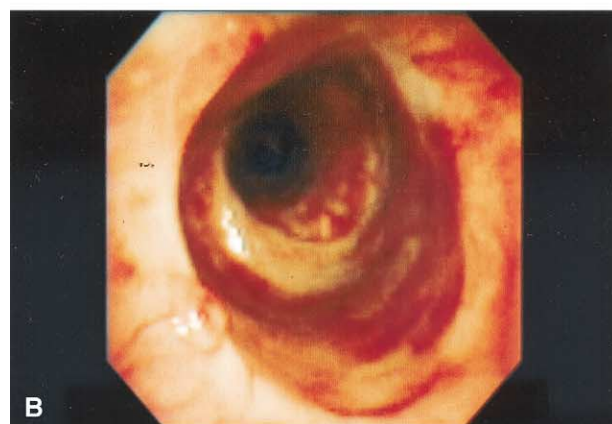
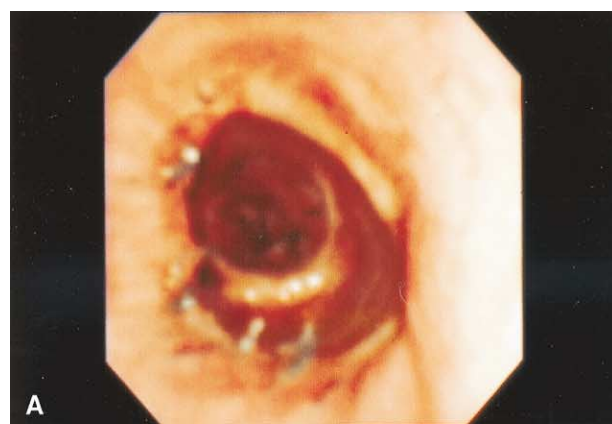


Figure 2. Bronchoscopic findings of the transplanted prosthesis. A, One week after surgery, erythematous and edematous change around the anastomosis were seen. B, One month after surgery, mild hypertrophy of the proximal anastomosis was observed. C, Two months after surgery, no stenosis was identified.

cytometer and a light microscope (Nikon, Kawasaki, Japan). PLGA scaffolds (6×8 cm) were prewetted with DMEM and seeded with $150 \mu\text{L}$ of a suspension of culture medium containing 4×10^6 cells. Cell-polymer constructs were incubated for a week in full keratinocyte culture medium containing 1% penicillin-

streptomycin under 5% CO₂ at 37°C until implantation into the peritoneal cavity. The medium was changed every other day.

In Vivo Culture of the Prosthesis

After the preparation of these implants, each animal was anesthetized as described above. The greater omentum was brought out of the peritoneal cavity via a small midline laparotomy. Each prepared autogenous tracheal prosthesis was wrapped with the lower portion of the greater omentum, fixed with several interrupted polypropylene 4-0 sutures, and replaced into the peritoneal cavity. The wound was sutured and the dog was allowed to recover.

Intrathoracic Tracheal Replacement

One week later, the same method of anesthesia was used to perform the tracheal implants. A second midline upper abdominal incision was used to free an adequate length of the greater omentum with its tracheal prosthesis. The left gastroepiploic blood vessels were ligated, divided at their origin from the splenic vessels, and dissected from the stomach wall. The omentum with its prosthesis was passed into the right thorax without torsion or tension via a 2-cm opening in the dome of the right diaphragm and the abdomen was then closed.

The trachea was then approached through a right posterolateral thoracotomy in the fourth intercostal space. After division of the azygos vein, the distal trachea was exposed. Ten distal tracheal cartilaginous rings (5 cm) were mobilized and resected from 6 cm to 1 cm above the carina. Ventilation and anesthesia were maintained via a new endotracheal tube passed through a sterile corrugated ventilating line. The tracheal prosthesis was raised into the operation field from the thorax and implanted in the resected trachea with interrupted 4-0 polyglactin 910 sutures. End-to-end anastomoses were performed, first distal and then proximal. The redundant omentum was used for loose wrapping of the proximal anastomosis.

A 24F drain tube was inserted into the thorax through a separate stab incision and the incision was closed. The chest drain was removed in the operating theater after the lung had been inflated. Anesthesia was stopped and the endotracheal tube was removed.

Postoperatively, each dog received an intravenous injection of 500 mg cefamezine on the day of the operation and 250 mg of cefamezine (intramuscular) per day for 3 days.

Postoperative Observations

Bronchoscopy was carried out periodically with the animals under general anesthesia. The luminal surface was observed with a bronchoscope (model BF1T240, Olympus Optical Co, Ltd, Tokyo, Japan) to evaluate the incorporation of each implanted prosthesis with its host tissue and to check on any complications such as exposure of the mesh framework or luminal stenosis.

Results

All animals resumed full activity within 12 hours except 2 with pneumothorax: 1 needed a reinsertion of the chest tube to expand the collapsed right lung, and the other normalized without any intervention. However, both regained full activity within 2 days. One of the initial 10 animals with a tracheal prosthesis implanted in its peritoneal cavity devel-

oped bowel protrusion through the wound site after 5 days. This animal was omitted from the experiment because we suspected graft infection (Table 1).

After 1 week of implantation in the peritoneal cavity with its omental wrapping, the tissue-engineered mucosa was well incorporated with the prosthesis and surrounding omentum, although some areas were still brittle. Connective tissue had formed on the peripheral bare area where the mucosa had not formed a complete lining, and this helped to maintain the air-tightness of the prosthesis.

Bronchoscopic Examination

Fiber optic bronchoscopic examination 1 week after surgery showed mild erythematous and edematous changes around the anastomoses. However, these did not obstruct the tracheal lumen and abated after 1 month in every dog. Mild hypertrophy of the proximal anastomosis was observed in 2 of 9 dogs at 1 month; however, there were no further changes at 2 months when the transplanted grafts were almost completely incorporated with the neighboring tracheal tissue (Figure 2). Every animal with intrathoracic tracheal replacement will be killed for the systematic gross and microscopic examination after 6 months of observation.

Discussion

Neville and colleagues¹⁰ stated that the ideal tracheal prosthesis should be airtight, of adequate consistency, and well tolerated by the host. It should also cause minimal inflammatory reactions but still be incorporated by the surrounding tissue. Moreover, it should be impervious to fibroblastic and bacterial invasion of the lumen and yet permit the ingrowth of respiratory epithelium along the lumen. We developed a novel tracheal prosthetic graft that may fulfill these requirements.^{8,9} The basic framework of the graft is of highly porous synthetic material that promotes the ingrowth of new vessels. The luminal surface of the original graft was lined with autogenous mucosa from the oral cavity to promote the epithelialization of the graft. A biodegradable glue such as gelatin was used to enhance the airtight attachment between tissue and prosthetics. We prepared the graft by transplanting it into the greater omentum to promote vascularization of the graft and to maintain the viability of the implanted mucosa. In these studies, we concluded that to prevent graft rejection, autogenous mucosa is critical in the development of an artificial trachea.

Initially, we tried to use buccal mucosa because we had good results from previous experiments. However, because of the limited size of the oral cavity of the dog and hence a limited number of cells, the preparation of the mucosa took too long (typically more than 6 or 7 weeks after the harvest). We therefore changed the source of epithelial cells from the buccal mucosa to the skin.

The use of skin for the source of epithelial cells may be controversial because it is unclear whether such epithelium will be suitable for the luminal surface of the airway. We plan to wait more than 6 months for the systematic histologic evaluation because we had observed that all the lining epithelium had been replaced with ciliated columnar epithelium as in the normal airway tract, even though originally it had been stratified squamous epithelium.⁹

In these experiments, we replaced experimental intrathoracic tracheal lesions with our prostheses. Contrary to cervical tracheal replacements, where the failure of a prosthesis would not be life-threatening, intrathoracic tracheal replacement has to be successful to save the patient's life. Any complications, such as minor dehiscence of the anastomosis or stenosis by ingrowth of granulation tissue, may hamper the activity of the recipient. Therefore, an implanted tracheal prosthesis should maintain its luminal status, diameter, and viability throughout life. In this study, all the dogs resumed full activity after replacement of the intrathoracic trachea and showed good tracheal anatomy. No animal showed anastomotic or central stenosis. We believe that maintenance of the viability of the cells on the tissue-engineered mucosa helps prevent the chronic infections that could lead to the formation of granulation tissues. Previous report on tracheal prostheses³ have shown that when prostheses longer than 5 cm were used there were complications such as mesh exposure and luminal stenosis. However, no such complication was noted in our experiments. Moreover, central stenosis, which is 1 of the most frequent complications associated with porous types of tracheal prostheses, was not a problem for our prosthesis.

There are several unanswered questions and drawbacks yet in this experiment. First of all, although we had good results in survival and postreplacement function of the tested animals, we do not know whether the implanted cells could maintain their viability and proliferative activity. From the previous experiment, we had observed that the cells had unaltered cellular morphology, suggestive of normal cell function.⁹ We plan to study systematic and serial gross and microscopic examination from 1 week to 6 months after the replacement. Such a study will help to know whether the graft was infected, caused inflammatory reaction to the surrounding tissue, and permitted the ingrowth of respiratory epithelium from the adjacent tracheal mucosa. Second, an incision is needed to obtain autogenous material, rather a long waiting time to establish the cell culture and lengthy placement of the prosthesis in the peritoneal cavity. We hope that technical development of tissue engineering will simplify the process and shorten the preparatory time. Third, literally speaking, this prosthesis is a hybrid graft undergoing graft regeneration following implantation¹¹ and is not an ultimate tissue-engineered tracheal prosthesis. Sometime in the future, we will have a

tissue-engineered tracheal prosthesis that can be readily available and simple to use.

However, we successfully performed long-segment thoracic tracheal replacement with immediate recovery of the animals and long-term stenosis-free trachea. These results also confirm the high biocompatibility of our current prosthesis. We conclude that although we need to confirm its long-term safety and biocompatibility, this tracheal prosthesis with its tissue-engineered skin cell-based mucosa appears promising for the repair of thoracic tracheal defects.

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Discussion

Dr Paolo Macchiarini (Hannover, Germany). Yesterday I had a wonderful discussion with Mr Garillo, and he asked me, "Paolo, do you know why I wrote these 2 editorials on tracheal transplantation?" I told him, "No idea." He said, "Well, I just wanted to avoid that we are still killing small or large animals."

I congratulate you, first of all, because this is a very nice study. However, I do have a few comments and some questions.

The comments are as follows. You are utilizing the same principle that has been described by others in the 70s and 80s. Since then, no one, except Eric Rose in 1971, has used the principle by simply taking off a segment of trachea, placing it in the muscles, sternocleidal muscle, and then implanting that oto-

topically at 2 weeks. We don't know if it worked, because there was no publication following the first one. My problem is that all the people who have already used clinically your support, which is polytetrafluoroethylene, 100% of the time see emesis, bleeding, infection, despite the use of indirect or direct revascularization. And it makes sense if you try to generate tissue-engineered trachea that is longer than 5 cm. But less than 5 cm, clinically, we do not need that. This is from a clinical point of view.

The questions are the following: First, you used the mucosa, or whatever, from the skin of the abdomen. From a methodological point of view, would it be not better to simply take off the blood samples and generate, as should be done in tissue engineering, this respiratory epithelium simply from fibroblasts? This is the first question.

Dr Kim. Actually this is 1 of a series of my experiments. At the time, as already I told you, I used the buccal mucosa from the oral cavity to make the mucosa line of the prosthesis. Originally the buccal mucosa had that squamous epithelium; but after 6 months of observation, the histology shows the epithelial cells change to columnar epithelium just like the respiratory epithelial cells. So I didn't check the histology yet, but I'm just waiting. But I expect it would be changed to the celiac columnar epithelium rather than just the growth of the fibroblasts.

You said there are several groups that have used PTFE graft, or mesh, but 1 of the most important things I think is the maintenance of the viability of the prosthesis. At the time they just wrapped the prosthesis with the omentum like that, because there was no mucosa. That is why they had more infection and dehiscence, I think.

Dr Macchiarini. The second question is, how are you sure that these are really the epithelium that you have engrafted, simply because if you transplant 5 cm of the trachea, you never know if this . . . well, we should know from clinical and experimental data, but still there is a migration from the proximal and lower stump from the respiratory cells over the graft. So before you state that, you should have made these biopsies, or at least a molecular biologist study, to be sure that they are in place.

And concerning the variability of the graft, I think, looking at the wonderful videos, just take care if you would like to transpose that in clinical practice, because I didn't see . . . for instance, the muscle, the trachea muscle, the posterior wall is simply cartilage or is whatever, just a circle. And if the patient or whatever needs to breathe, then it will never work with the intrathoracic mechanics.

Dr Kim. That's quite a good point. In my previous experiments, you know, previously several Japanese groups reported that

there is some migration of the epithelial cells from the native trachea, but the extent was less than 2 cm. Usually it's just 1 cm or less.

The reason why I used the 5-cm Rastelli replacement is to watch whether there is the migration of the epithelial cells or just a change of the epithelial cells. In my previous experiments, all the epithelial cell layers, all the mucosal cells changed to respiratory columnar epithelium, not just the anastomosis site. So yes, the comment is quite good. Maybe I can use molecular biologist techniques or something like that. But by histologic evaluation, I think those cells are changing because of the respiratory environment.

Dr Douglas E. Wood (Seattle, Wash). I just wanted to make sure I understood the sequence in your experimental model. This model required 2 laparotomies, is that right? First, for harvesting the epithelium and placing the prosthesis in the omentum, and then a second laparotomy for transposition of the omentum and prosthesis up to the neck or chest.

Dr Kim. Yes. The first laparotomy is just harvesting of the skin. But there are 2 laparotomies needed, the first just to put the prosthesis in the omentum. And after a week, a week later, I use the replacement of the prosthesis with fat by omental vessels to the thoracic trachea.

Dr Wood. And have you noted any problem with mobility of the omentum a week after it's been manipulated and the prosthesis placed in it?

Dr Kim. In my motion picture, you saw the wrap. The reason why I used this is that after a week sometimes the omentum adheres and sometimes there is some limitation of the mobilization. That is why I wrapped in the first experiment in an abdominal procedure.

Dr Wood. I guess I am wondering whether you think that one might be able to skip a step and actually do it as a 1-stage operation where the prosthesis, skin harvest, and omental wrapping is done in situ at the same time as the tracheal replacement?

Dr Kim. I hope so, but I did not try it yet.

Dr Mark S. Allen (Rochester, Minn). Is there some problem with making the initial graft a cylinder instead of a flat piece of polypropylene? If you made it a cylinder initially, then you wouldn't have to fold it around and manipulate it.

Dr Kim. With the omentum, it's really difficult to make a good anastomosis when I do the thoracic tracheal replacement. That is why I just open it and make a round tube when I do the thoracic tracheal replacement.